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Contributed Paper

Xylitol Production by Thermotolerant Methylotrophic Yeast *Ogataea siamensis* and Its Xylose Reductase Gene (*XYL1*) Cloning

Wanatchabhorn Boontham [a], Nantana Srisuk*[a,b], Kanya Kokaew [a], Pantida Treeyoung [a], Savitree Limtong [a,b], Arinthip Thamchaipenet [b,c] and Hiroya Yurimoto [d]

[a] Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
[b] Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.
[c] Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
[d] Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan.
*Author for correspondence; e-mail: fscints@ku.ac.th

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ABSTRACT

Thermotolerant methylotrophic yeast, *Ogataea siamensis* N22, possesses high yield of xylitol from D-xylose at high temperature. Xylitol production was compared at 37°C and 40°C in a 2.5L fermenter with 1.5% (v/v) methanol and 100 g/L D-xylose supplementation. The maximum yield of xylitol ($Y_{p/s}$) and volumetric productivity (Q_p) were 0.83 g/g and 0.36 g/L/h at 37°C, respectively; while at 40°C the values appeared to be 0.87 g/g and 0.34 g/L/h, respectively. The yeast strain was subjected to characterization for *XYL1* gene coding for xylose reductase which catalyzes an initial step of xylitol production from D-xylose. *XYL1* gene was cloned from *O. siamensis* N22 genomic DNA and subsequently sequenced. As a result, an open reading frame of 960 bp encoding 319 amino acids was obtained with a predicted protein molecular mass of 36.3 kD. Alignment of deduced amino acids sequence indicated that xylose reductase of *O. siamensis* N22 was closely related to aldose reductase of *Candida boidinii* with 72% similarity. *O. siamensis* N22 recombinant carrying an extra copy of *XYL1* integrated into the chromosome showed slightly higher xylose reductase activity and hence xylitiol production than that of the original host strain.

Keywords: *Ogataea siamensis*, xylitol production, xylose reductase, gene cloning

1. INTRODUCTION

Xylitol is a five-carbon sugar alcohol naturally found in many fruits and vegetables with minor quantity. Despite of the same sweetness level as sucrose, xylitol possesses a reduced calorie sweetening power as well as non- and anti-cariogenic properties [1]. Another beneficial health property of xylitol has been found fascinating to all diabetics as insulin is not required for its metabolism [2]. Broad applications of xylitol have long been

reported in pharmaceuticals and food industries. These include the production of chewing gum as a result of its cooling effect and negative heat of solution [3]. It is also commonly used in production of sugar free oral tablets and oral hygiene products. Alternatively, xylitol has been applied as an alternative preservative for cosmetic products [4]. It was claimed that xylitol shared an increasing market value up to $340 million with 43,000 tons of global xylitol consumption in 2005 [5].

Industrial production of xylitol is achieved by a nickel-catalyzed hydrogenation of D-xylose which is costly and energy consumed [1]. These disadvantages are overcome by a more environmental friendly biological process using xylose-assimilating microorganisms. Microbial strains able to potentially assimilate xylose to xylitol have been screened and used as a tool to fulfill this approach. Among microbes able to assimilate xylose to xylitol, yeast appears to be an organism of choice that xylitol production of various genus and species has been investigated such as *Debaryomyces hansenii* [5], *Candida tropicalis* [6-7], *C. guilliermondii* [8], *Pichia guilliermondii* [9] and methylotrophic yeasts, extensively focused on *C. boidinii* [10]. Yeast strains those keen on xylitol production succeeded to employ xylose reductase, the first enzyme in xylose metabolic pathway, to utilize xylose as their substrate for growth.

Xylose reductase, XR, (EC. 1.1.1.21) is responsible for reduction of D-xylose to xylitol with concomitant NAD(P)H oxidation. XR is important in fermenting xylose to xylitol. Yeast XRs were categorized into the aldose reductase (ALR) family, a member of the aldo-ketoreductase (AKR) superfamily, based on sequence and structural similarities [11]. The enzymes were also classified into two groups according to their coenzyme specificity [12]. Generally, XR is specific for NADPH, as cofactor such as that from *Candida intermedia*, *C. guilliermondii* and *Debaryomyces hansenii* UFV-17 [13-15]. However, both NADH and NADPH are usable for yeasts *Pichia stipitis*, *C. tenuis* and *C. shehatae* [16-18].

Several yeast XR genes have been cloned, characterized and expressed including *Kluyveromyces lactis* [21], *C. tropicalis* [22], *C. guiliermondii* [23], *C. parapsilosis* [20], *C. boidinii* [19], *Pachysolen tannophilus* [24] and *Pichia stipitis* [25] using different methods. However, up to date, neither xylitol production nor xylose reductase enzyme from *Ogataea siamensis* has not yet been reported.

It has been well agreed that using thermotolerant microorganisms would reduce the cooling cost during industrial fermentation process especially in tropical countries. We therefore report here xylitol production of *O. siamensis* N22 at high temperatures i.e. 37°C and 40°C. In addition, we cloned and characterized *O. siamensis* N22 *XYL1* gene encoding xylose reductase, the first enzyme catalyzing the reduction of D-xylose to xylitol in xylose assimilating pathway.

2. MATERIALS AND METHODS

2.1 Yeast Strain and Xylitol Production

Thermotolerant yeast *Ogataea siamensis* N22 was maintained in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) with or without 1.5% agar depending on purpose of cultivation. For xylitol production, yeast culture was grown in basal medium [10] supplemented with 1.5% (v/v) methanol, 10g/L xylose, 5 g/L casamino acid and 0.5 g/L $MgSO_4$. $7H_2O$ at pH 7.0 in a 2.5 L bioreactor (Biostat B, Braun Biotech International, Germany) with aeration rate 1.75 vvm and agitation speed 150 rpm.

2.2 Growth, Xylose and Xylitol Determination

Yeast growth was assessed as cell dry weight. Xylose and xylitol were analyzed by HPLC (Hewlett Packard1100, Germany) with RI detector and a sugar column (ULTRON PS-80, Shinwa Chemical Industries, Kyoto, Japan).

2.3 *XYL1* Gene Cloning and Sequencing

The genomic DNA of *O. siamensis* N22 was prepared [26] and partially digested by *Bsp*143I (Fermentas, USA). The DNA library was constructed using pJET1.2/ blunting vector (Fermentas, USA) and introduced into *E. coli* XL-1 Blue. Recombinants were grown on LB agar containing 100 μg/mL ampicillin and screened by PCR amplification using primers OSXRf55 (5'- TTYGGCTGYTGGAAAGTCG -3') and OSXRr57(5'- YTTKSYTGGGTCAAG TATGGRTGRTG -3') specifically designed for XR. Polymerase chain reaction (PCR) was performed in a 25 μL reaction mixture containing 5ng genomic DNA template (2 μL), 2.5 μL 10x PCR Buffer (25 mM $MgCl_2$), 0.6 μL dNTP mixture (2.5 mmol/ L), 2 μL primer mixture (1pmol/L), 0.13 μL Taq DNA polymerase (5 U/L; Fermentas, USA) and 15.8 μL distilled water. The PCR conditions were as follow: 3cycles of 1 min at 94.0°C, 1 min at 64.0°C, and 1 min at 72.0°C, preceeded by 5 min at 94.0°C and followed by 1min at 72.0°C. The positive clones were sequenced (First Base Inc., Malaysia) and analyzed.

2.4 Multiple Alignment and Phylogenetic Analysis of Protein Sequence

Nucleotide and amino acid sequences similarity as well as sequences identity were determined using BLAST programs [27]. Amino acid sequence alignment was performed using ClustalW2 program [28]. A phylogenetic tree was generated from the alignments using the neighbor-joining method [29] and bootstrap analysis was sampled 1,000 times.

2.5 Characterization of *XYL1* Putative Protein

The putative protein of *XYL1* was determined using ORF Finder program http://www.ncbi.nlm.nih.gov/projects / gorf). The predicted protein molecular mass was achieved using Compute pI/Mw (http://web.expasy.org/compute_pi). Various yeast XR amino acid sequences and the deduced amino acid sequence of *Ogataea siamensis* N22 XR were aligned and the putative active site was determined using ScanProsite program (http:// au.expasy.org/tools/scanprosite/). Nucleotide sequence of *O. siamensis* N22 *XYL1* coding region was deposited in GenBank, accession no. FJ763639.

2.6 Construction and Analysis of *Ogataea siamensis* N22 Overexpressing *XYL1*

The *O. siamensis* N22 *XYL1* coding region was amplified from genomic DNA by PCR using specific primers OSXRf-78 (5'- GCTTCGAATATTGTGTGGGGAATTTCTTG -3' with the underlined *Sfu*I site for other purpose) and OSXRr95 (5- CGGAATTC GGCCTTTATTTACTGTTTCTAG-3 with the underlined *Eco*RI site) under the same PCR condition described above. PCR product was purified and inserted at *Eco*RI site of pGAPZ B vector (Invitrogen, USA), an integrative vector containing Zeocin resistant marker and a constitutive promoter, P_{GAP}. The resulting plasmid, pGAPXR, was then transformed into sorbitol treated competent yeast cell by Bio-Rad gene pulser (Bio-Rad, USA) at 25 μF and 2.5 KV. Using similar technique, the PCR product was purified and inserted

into pPICZ B vector (Invitrogen, USA), an integrative vector containing Zeocin resistant marker and a regulated promoter, P_{AOX}. The resulting plasmid pPICXR was transformed into competent *O. siamensis* N22 cells using the same procedure that yielded pGAPXR. Transformants were selected on YPD agar supplemented with 5μg/mL Zeocin™ (Invitrogen, USA). *O. siamensis* N22 recombinants containing the construct integrated into the chromosome were investigated for their XR activity in comparison to the original host strain by cultivation in 50 mL of YPX medium (1% yeast extract, 2% peptone and 2% xylose) on 170 rpm rotary shaker at 37°C for one day. Cells were harvested and resuspended in extraction buffer prior to cell breakage.

2.7 Xylose Reductase Activity Assay

Cell crude extracts were prepared using glass beads and mini-bead beater (Biospec, UK). XR activity in cell crude extracts was determined spectrophotometrically [20]. NADH was used, instead of NADPH, to determine cofactor preference of *O. siamensis* N22 XR activity. Protein concentration in cell extracts was determined by Bradfords mini assay [20]. All assay experiments were performed in triplicates.

2.8 Xylitol Production by Yeast Transformants

Ogataea siamensis N22 and transformants were cultivated in 50 mL of basal medium [10] supplemented with 1.5% (v/v) methanol, 100 g/L xylose, 5 g/L casamino acid and 0.5 g/L $MgSO_4$. $7H_2O$ at pH 7.0 in a 250 mL flask with shaking speed 150 rpm for 14 days. Samples were taken for determination of growth every day whereas xylose and xylitol concentration were assessed every two days.

3. RESULTS AND DISCUSSION

3.1 Thermotolerant *Ogataea siamensis* N22

Ogataea siamensis (formerly *Pichia siamensis*) is a methylotrophic yeast isolated from tree exudates in Thailand and was taxonomically validated earlier [31-32]. *O. siamensis* N22 is claimed as a thermotolerant yeast strain according to higher specific growth rate appeared at 30°C when compared to the value observed at 40°C. Moreover, growth on YPD agar at 8°C has been observed (data not shown). This is in consistent to previous report on characteristics of thermotolerant yeast [33].

3.2 Xylitol Production in 2.5L Fermenter

Thermotolerant methylotrophic yeast *Ogataea siamensis* N22 was grown in 2.5L bioreactor under aerobic batch cultivation and xylitol production at 37°C and 40°C (Figure 1). Xylose was completely used up within 10-11 days of cultivation in accordance with observed stationary phase growth. The maximum yeast growth at 40°C was slightly lower (14.4 g/L) than that observed at 37°C (15 g/L). However, comparative maximum xylitol concentration of 82.8 g/L and 80.8 g/L were observed after 11 days of cultivation at 37°C and 40°C respectively. The theoretical xylitol production yield from xylose ($Y_{p/s}$) as well as volumetric productivity (Q_p) were calculated. Results exhibited the maximum yield of xylitol production and volumetric productivity of 0.83 g/g and 0.36 g/L/h at 37°C while the values appeared to be 0.87 g/g and 0.34 g/L/h respectively at 40°C (Table 1). Interestingly, the higher maximum yield was observed within 8 days of cultivation at 40°C, whereas the maximum yield at 37°C was not observed until the cultivation reached 11 days.

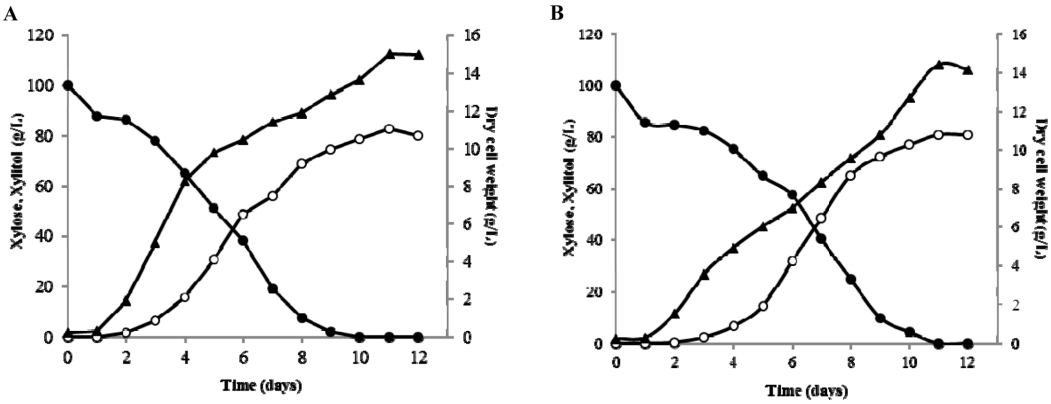

Figure 1. Xylitol production by *Ogataea siamensis* N22 in basal medium supplemented with 1.5% methanol, 100 g/L xylose, 5 g/L casamino acid and 0.5 g/L $MgSO_4.7H_2O$ with aeration rate of 1.75 vvm, agitation speed of 150 rpm and the fermentation temperature were set at 37°C (A) and 40°C (B). Symbols: O xylitol ; ● xylose ; ▲ dry cell weight.

Table 1. Xylitol production in batch cultures of *Ogataea siamensis* N22 at 37°C and 40°C in 2.5 L bioreactor with aeration rate of 1.75 vvm and agitation speed of 150 rpm.

Fermentation temperature (°C)	Fermentation period (day)	Residual xylose[a] (g/L)	Xylitol concentration[a] (g/L)	Xylitol yield (g/g xylose)	Volumetric productivity (g/L/h)
	0	100	0	-	-
	1	88	0	-	-
	2	86.5	1.6	0.12	0.03
	3	77.9	6.6	0.30	0.09
	4	65.1	15.8	0.45	0.16
37	5	51	30.5	0.62	0.25
	6	38.2	48.7	0.79	0.34
	7	19.2	55.8	0.69	0.33
	8	7.5	68.7	0.74	0.36
	9	2	74.6	0.76	0.35
	10	0	78.6	0.79	0.33
	11	0	82.8	0.83	0.31
	12	0	80	0.80	0.28
	0	100	0	-	-
	1	85.9	0	-	-
	2	84.8	0.4	0.03	0.01
	3	82.5	2.4	0.14	0.03
	4	75.5	6.6	0.27	0.07
40	5	64.8	14.5	0.41	0.12
	6	57.5	31.5	0.74	0.22
	7	40.3	48.3	0.81	0.29
	8	24.8	65.1	0.87	0.34
	9	9.8	72.4	0.80	0.34
	10	4.3	76.9	0.80	0.32
	11	0	80.8	0.81	0.31
	12	0	80.7	0.81	0.28

[a] The values were the means of three independent samples.

Results appeared that, even at high temperature as 40°C, *O. siamensis* N22 could produce higher xylitol concentration than that has been reported in other methylotrophic yeasts. After 5 days of cultivation, *C. boidinii* No. 2201 grown in xylose (10 g/L) medium supplemented with 2% (v/v) methanol showed only 48.5 g/L xylitol at 28°C [34] whereas, under the same xylose content and 1.5% (v/v) methanol supplementation, *O. siamensis* N22 produced 55.8 g/L and 65.1 g/L xylitol after 7 and 8 days of cultivation at 37°C and 40°C, respectively. *Hansenula polymorpha* DL1 was shown to give high yield of xylitol as 57 g/L xylitol from medium plus 110 g/L D-xylose and 1% (v/v) methanol after 3 days of 30°C cultivation [10]. Although these two methylotrophic yeasts seem to produce xylitol at earlier fermentation time than *O. siamensis* N22, it should be, however, noted that we presented here the xylitol production at higher temperature i.e. 37°C and 40°C. The other thermotolerant yeast, *Debaryomyces hansenii*, showed the maximum xylitol produced by free cells as 68.6 g/L xylitol at 40°C from 100 g/L of xylose, with a yield of 0.76 g/g after 7 days of cultivation [9]. *O. siamensis* N22, although at longer period of incubation, obviously showed higher xylitol yield than that reported from *D. hansenii*. This therefore confirms that *O. siamensis* N22 has the ability to produce xylitol from D-xylose at high temperature.

Xylitol production in methylotrophic yeast was significantly increased when supplemented with methanol. This had been proposed to be due to the oxidation of methanol that supplies NADH to enhance the reduction of D-xylose [34]. However, this explanation may not be applied to our *O. siamensis* N22 and its transformant since we eventually found that NADPH was more preferable than NADH. The highest xylitol production yield of *O. siamensis* N22 observed at 37°C (0.83 g xylitol/g xylose consumed) appeared to be comparable to the highest value reported in *Candida* sp. [10] and higher than those previously reported [35].

3.3 Cloning and Sequencing of *XYL1* Gene from *Ogataea siamensis* N22

Partially *Bsp*143I digested genomic DNA of *O. siamensis* N22 was purified and selected for the DNA fragment size about 1-3 kb for cloning into pJET1.2/blunt vector. The recombinant plasmids harboring XR gene (*XYL1*) were then screened by PCR using specific primers, OSXRf55 and OSXRr570. Nucleotide sequence analysis of the positive clone showed a full-length 960 bp of *XYL1* gene encoding a putative 319 amino acids as well as the potential promoter element containing a TATA-like sequence ($^{-112}$TATAA^{-108}) upstream from start codon and a polyadenylation signal (^{974}AATAAA^{979}) downstream from stop codon (Figure 2). This consistently appeared in the reports on the existence of TATA-like sequence and polyadenylation signal appeared in yeast genes responsible for xylose metabolism [21-22].

Amino acids sequence of *O. siamensis* N22 XR was examined and the active site consists ^{269}IPKSNQKERLLQNLSV^{284} indicating the signature of yeast XR active site, ^{269}IPKS^{272} , was identified (Figurre 2) [22, 24]. Multiple alignment of the deduced amino acid sequences of *O. siamensis* N22 XR and other yeasts revealed the conserved amino sequence of aldo/ketoreductase family putative active site signature i.e. IPKS (Figure 3). Molecular mass of *O. siamensis* N22 XR was predicted from deduced amino acids sequence as 36.3 kD. This prediction on XR molecular mass well agrees with that reported for

Candida tropicalis XR [22]. Phylogenetic analysis of XR homologs suggested that *O. siamensis* N22 XR is closely related to aldose reductase of *Candida boidinii* (Figure 4). Both results show close relationship between XR of *O. siamensis* N22 and two members of the genus *Candida*. However, it should be noted that, *O. siamensis* N22 was formerly classified as a member of the genus *Pichia* [31-32]. More details on enzyme properties may be needed to rule out the concrete relation of *O. siamensis* N22 XR and other yeast XRs.

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-287                                CTTTGGAATTGTTCTTCCAGGGCCGCCACCGCCCT
-252 TGGTAATTTCCATCCTTTTAAAGGCCATGATACCCATTTTTAAAATTCCGAATAAGAATTATT
-189 TTTGTCGAAAAATCTAACTTCCCACCTCCCCACCTTTGACCCCGAATTTTCGCTATTGGGGCG
-126 GTGAGGATATTCTTTATAAAGTTTCCAGCTGAAGTAGCAATCTTGAGATATTGTGTGGGGAAT
 -63 TTCTTGGAAAAAAACCCTTACTGGAATTTATTTTTCAACCACCTTATTATTTCTTGAAAGAAA
   1 ATG GTT TCT AAA ACA GTG AAG TTG AAC AAT GGC ATT GAG ATC CCA GTA
   1  M   V   S   K   T   V   K   L   N   N   G   I   E   I   P   V
  49 GTT GGA TTT GGC TGC TGG AAA GTC GAA AAG TCA ATC TGC GCT GAC CAG
  17  V   G   F   G   C   W   K   V   E   K   S   I   C   A   D   Q
  97 ATT TAC GAA GCT ATC AAA GTC GGA TAC CGT CTG TTC GAT GGC GCT ATG
  33  I   Y   E   A   I   K   V   G   Y   R   L   F   D   G   A   M
 145 GAT TAT GGT AAT GAG AAG GAA ATT GGT GAA GGC GTT GCT AGA GCA ATC
  49  D   Y   G   N   E   K   E   I   G   E   G   V   A   R   A   I
 193 AAA GAT GGA TTG GTT AAG AGA GAG GAA TTG GTT ATT GTC TCA AAG TTG
  65  K   D   G   L   V   K   R   E   E   L   V   I   V   S   K   L
 241 TGG AAC AGT TAT CAT CAT CCT GAT AAC GTG AAG AAG GCA ATT AGA AGA
  81  W   N   S   Y   H   H   P   D   N   V   K   K   A   I   R   R
 288 GTT CTG GAT GAC TTG AAG CTT GAC TAT CTC GAC ATC TTT TAC ATC CAT
  97  V   L   D   D   L   K   L   D   Y   L   D   I   F   Y   I   H
 337 TTT CCT GTC GCT CAA AAA TTT GTG CCA TTT GAA GAG AAG TAT CCT CCA
 113  F   P   V   A   Q   K   F   V   P   F   E   E   K   Y   P   P
 385 GGG TTG TAC TGT GGT CCT AAT GGA TGG GAG TAT GAA GAC GTT CCT TTA
 129  G   L   Y   C   G   P   N   G   W   E   Y   E   D   V   P   L
 433 GCA GTC ACA TGG AAG GCT ATG GAA GGT TTA GTT GAA GAA GGT CTT GTT
 145  A   V   T   W   K   A   M   E   G   L   V   E   E   G   L   V
 481 AAA TCG ATT GGT ATC TCT AAC TTC TCT GGA GCA CTC ATT CAG GAC CTG
 161  K   S   I   G   I   S   N   F   S   G   A   L   I   Q   D   L
 529 TTG AGA GGA TGC AAG ATT AAG CCC CAA TTG CTT CAA ATT GAA CAC CAT
 177  L   R   G   C   K   I   K   P   Q   L   L   Q   I   E   H   H
 577 CCA TAC TTG ACC CAG GAA AGA TTG GTC AAG TAT GTT CAG GCT CAA GAT
 193  P   Y   L   T   Q   E   R   L   V   K   Y   V   Q   A   Q   D
 625 ATT GCA GTT GTG GCT TAC TCC TCA TTC GGC CCG CAA TCA TTT GTT GAA
 209  I   A   V   V   A   Y   S   S   F   G   P   Q   S   F   V   E
 672 CTT GAT CAT GCC AAG GCC AAA GAC ACC GTT TCC CTA TTG AAG CAC GAA
 225  L   D   H   A   K   A   K   D   T   V   S   L   L   K   H   E
 721 ACC ATT AAC TCA ATT GCT TCG GCT CAT AAG GTT TCA CCT GCT CAA GTG
 241  T   I   N   S   I   A   S   A   H   K   V   S   P   A   Q   V
 769 CTG CTC AGA TGG GCA ACC CAA AGA AAC GTA TTG GTT ATT CCA AAG TCT
 257  L   L   R   W   A   T   Q   R   N   V   L   V   I   P   K   S
 817 AAC CAG AAG GAA AGA TTA CTG CAG AAC TTA TCG GTG AAC GAT TTC AAT
 273  N   Q   K   E   R   L   L   Q   N   L   S   V   N   D   F   N
 865 TTG AGT GAG AAA GAG ATT AAA GAA ATC AGT GCT TTA AAT CAG GAT CTT
 289  L   S   E   K   E   I   K   E   I   S   A   L   N   Q   D   L
 913 AGA TTT AAT GAT CCT TGG ACT TGG GAT GAA ATT CCA ACC TTC ATT TAA
 305  R   F   N   D   P   W   T   W   D   E   I   P   T   F   I   *
 961 ACTAGAAACAGTAAATAAAGGCCGAATTCCG
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Figure 2. Nucleotide and deduced amino acid sequences of XR gene (*XYL1*) of *Ogataea siamensis* N22. An asterisk (*) indicates stop codon. The deduced TATAA promoter site is underlined (thick line). The AATAA putative polyadenylation signal is also underlined (thin line). Amino acid sequence in the gray box show aldo/ketoreductase family putative active site signature.

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Candida albicans      IADKHGKSPAQVLLRWATQRNIAVIPKSNNPDRLAQNLAVVD-FDLTEEDLQAISKLDIG 355
C. dubliniensis       IADKHGKSSAQILLRWATQRNIAVIPKSNNPDRLAQNLAVVD-FDLTDEDLQAISKLDIG 315
C. tropicalis         IADKHGKSPAQVLLRWATQRNIAVIPKSNNPERLAQNLSVVD-FDLTKDDLDNIAKLDIG 308
Candida sp. GCY2005   IAEKHGKTPAQVLLRWATQRNIAVIPKSNNPARLAQNLSVVD-FDLSKEDIQEISALDIG 305
C. parapsilosis       IASKHKKSSAQVLLRWATQRGIAVIPKSNNPDRLAQNLNVSD-FELSKEDLEAINKLDKG 301
M. guilliermondii     VAGKVKKTPAQVLLRWATQRGLAVIPKSNNPDRLLSNLKVND-FDLSQEDFQEISKLDIE 301
D. hansennii          IANKNKKTPAQVLLRWASQRNIAVIPKSNNPDRLLQNLEVND-FDLSKEDFEEISKLDQE 301
C. shehatae           IAAKHNKVPAEVLLRWSAQRGIAVIPKSNLPERLVQNRSFND-FELTKEDFEEISKLDIN 307
C. tenuis             IAAKYNKTPAEVLLRWAAQRGIAVIPKSNLPERLVQNRSFNT-FDLTKEDFEEIAKLDIG 306
P. stipitis           IAAKHGKSPAQVLLRWSSQRGIAIIPKSNTVPRLLENKDVNS-FDLDEQDFADIAKLDIN 302
O. siamensis N22      IASAHKVSPAQVLLRWATQRNVLVIPKSNQKERLLQNLSVND-FNLSEKEIKEISALNQD 303
Candida boidinii      IASAHDVPPAKVLLRWATQRGLAVIPKSNKKERLLGNLKIND-FDLTEAELEKIEALDIG 304
Kl. lactis            IASKHKVTPQQVLLRWATQNGIAIIPKSSKKERLLDNLRINDALTLTDDELKQISGLNQN 311
                      :*      . ::*****::*..: :****.   **  *  .   : * . ::  *  *:
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Figure 3. Amino acid sequences alignment of 12 yeast xylose reductases and the deduced amino acid sequence of *Ogataea siamensis* N22 xylose reductase. A putative active site signature was shown as underlined.

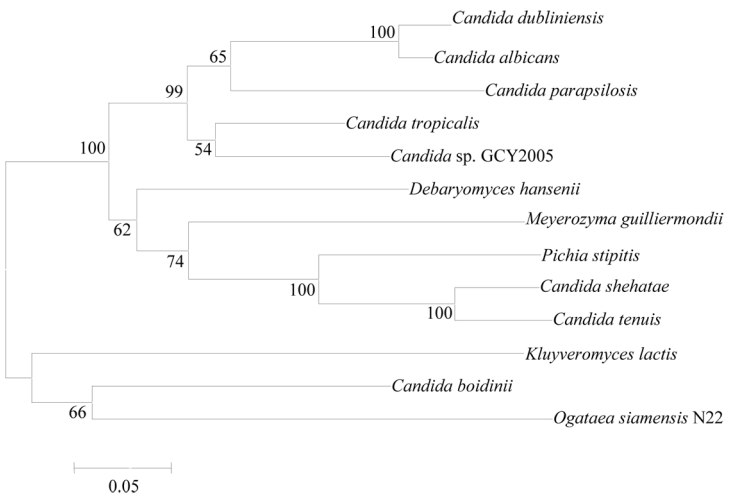

Figure 4. Phylogenetic tree of *Ogataea siamensis* N22 xylose reductase and other homologous xylose reductase reported in yeasts. A phylogenetic tree was constructed by Neighbor joining algorithm and the aligned regions after excluding sequences creating gaps. Boot strap values are shown on the branches and expressed in percentages.

3.4 Xylose Reductase Activity and Xylitol Production of *Ogataea siamensis* N22 Recombinants

A copy of *O. siamensis* N22 *XYL1* gene was expected to integrate into its chromosome using pGAPZ B vector. Three recombinants showed 0.5 fold higher XR specific activity (90-93 mU/mg protein) than that of the original host (60 mU/mg protein) while the strain habouring naked pGAPZ B showed equal level (61 mU/mg protein). We also constructed *O. siamensis* N22 transformants which was expected to possess extra copies of *XYL1* gene into yeast genome via another integrative vector carries regulated promoter P_{AOX}. However, two recombinants carrying extra copies of *XYL1* gene into their genome via pPICZ B vector also showed only 0.5 fold increased in XR specific activity. This unpleasant outcome may be due to inappropriate promoters used in this study i.e. P_{GAP} and P_{AOX} in pGAPZ B and pPICZ B, respectively. Expression of the *XYL1* gene on its own promoter may be a successive

task to solve this obstruct. Noticeably that XR activity of the original host appeared to be closed to that of *C. boidinii* (78 mU/mg protein) but only half of that of *C. tenuis* (150 mU/mg protein) [36]. Review on yeast XR activity with NADPH as cofactor has been shown to be various between 110 to 6430 mU/mg protein [37]. Investigation of cofactor preference revealed that NADPH was more favorable to *O. siamensis* N22 xylose reductase reaction than NADH.

We studied on optimal temperature for XR activity of *O. siamensis* N22 and its transformants at 30°C, 37°C and 40°C (data not shown). Results revealed that *O. siamensis* N22 and the two transformants carrying pPICZ B:: *XYL1* or pGAPZ B:: *XYL1* in their genome showed similar results i.e increased XR activity as the temperature increased up to 40°C.

Although only slight increase in XR activity observed, yeast growth as well as xylose and xylitol in culture medium were compared among *O. siamensis* N22 and the two transformants, one with pPICZ B:: *XYL1* and the other with pGAPZ B:: *XYL1* in the genome (Figure 5). Results indicated that *O. siamensis* N22 possessed slightly lower level of growth than that found in the two transformants. This therefore agreed with xylose consumption and xylitol production of *O. siamensis* N22 that appeared to be slightly lower than that of the two transformants.

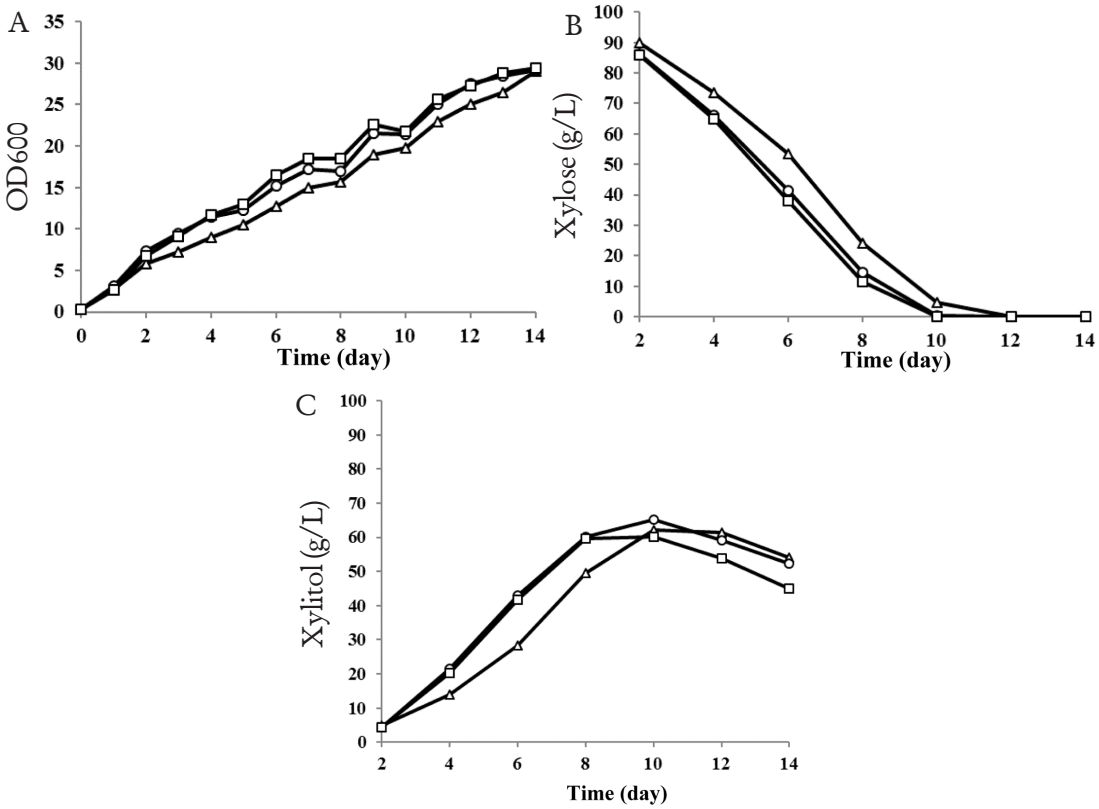

Figure 5. Growth (A) of *Ogataea siamensis* N22 and transformants, xylose (B) and xylitol (C) concentration in 50 mL of basal medium supplemented with 1.5% methanol, 100 g/L xylose, 5 g/L casamino acid and 0.5 g/L $MgSO_4$. $7H_2O$ in 250 mL Erlenmeyer flask under shaking speed 150 rpm at 37°C for 14 days. Symbols: Δ *O. siamensis* N22; ○ *O. siamensis* N22 with pGAPZ B:: *XYL1*; □ *O. siamensis* N22 with pPICZ B:: *XYL1*.

4. CONCLUSIONS

We describe here a report on xylitol production at high temperature of *Ogataea siamensis* N22, thermotolerant methylotrophic yeast isolated in Thailand. Xylitol production by *O. siamensis* N22 at 40°C appears to be promising since high xylitol production yield was obtained. The process optimization is considered to further obtain the highest yield as well as the shortest time of fermentation. We also publish here the nucleotide sequence of *O. siamensis XYL1* and attempt to overproduce xylose reductase enzyme in *O. siamensis* N22. Although high level of XR overproduction was not successively obtained, experimental scheme could be drawn for further fruitful research goal.

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